The Dendritic Cell Receptor DNGR-1 Promotes the Development of Atherosclerosis in Mice

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ABSTRACT

***Rationale*:** Necrotic core formation during the development of atherosclerosis is associated with a chronic inflammatory response and promotes accelerated plaque development and instability. However, the molecular links between necrosis and the development of atherosclerosis are not completely understood. C-type lectin receptor (Clec9a) or DNGR1 is preferentially expressed by the CD8α$^+$ subset of dendritic cells (CD8α$^+$DCs) and is involved in sensing necrotic cells. We hypothesized that sensing of necrotic cells by DNGR1 plays a determinant role in the inflammatory response of atherosclerosis.

Objective: We sought to address the impact of either total, bone marrow (BM) or CD8α$^+$ DC-restricted deletion of DNGR1 on atherosclerosis development.

***Methods and Results*:** We show that total absence of DNGR1 in apolipoprotein e-deficient mice (*Apoe*$^{-/-}$) mice as well as BM-restricted deletion of DNGR1 in low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice significantly reduce inflammatory cell content within arterial plaques and limit atheroslerosis development in a context of moderate hypercholesterolemia. This is associated with a significant increase of the expression of interleukin 10 (IL10). The atheroprotective effect of DNGR1 deletion is completely abrogated in the absence of BM-derived IL10. Furthermore, a specific deletion of DNGR1 in CD8α$^+$ DCs significantly increases IL10 expression, reduces macrophage and T cell contents within the lesions, and limits the development of atherosclerosis.

***Conclusions*:** Our results unravel a new role of DNGR1 in regulating vascular inflammation and atherosclerosis, and potentially identify a new target for disease modulation.

Keywords:
DNGR1, IL-10, CD8α$^+$dendritic cells, atherosclerosis, inflammation, necrosis.

Nonstandard Abbreviations and Acronyms:

Apoe$^{-/-}$	apolipoprotein e-deficient mice
BM	bone marrow
Clec9a	c-type lectin receptor
DAMPs	damage-associated molecular patterns
DC	dendritic cell
DNGR-1	Dendritic cell NK lectin Group Receptor-1
HFD	high fat diet
Ldlr$^{-/-}$	low-density lipoprotein receptor-deficient
MERTK	myeloid-epithelial-reproductive tyrosine kinase
MFGE8	milk fat globule EGF-like Factor 8
PRR	pattern recognition receptors
SYK	spleen tyrosine kinase

INTRODUCTION

Accumulation of apoptotic cells and necrotic debris within the lipid core is a major feature of advanced atherosclerotic lesions and has been associated with increased susceptibility to thrombotic complications following plaque rupture [1-4]. We and others have shown that accumulation of apoptotic and secondary necrotic debris in atherosclerotic lesions results from defective efferocytosis or clearance of apoptotic cells[3-5]. This in turn leads to activation of immune-inflammatory responses and accelerates the progression of atherosclerosis [3, 5, 6]. Mouse models of decreased efferocytosis, such as mice lacking the receptor myeloid-epithelial-reproductive tyrosine kinase (MERTK) or the bridging molecule milk fat globule EGF-like Factor 8 (MFGE8) showed an alteration of the protective immune response, associated with increased vascular inflammation and enhanced necrotic core formation [7, 8, 9]. However, the mechanisms involved in necrosis-induced inflammation during atherosclerosis development are not fully understood.

Dying cells release endogenous molecules known as damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors (PRR) in immune cells. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are 2 main PRR families expressed on immune cells, including macrophages and dendritic cells (DCs). Dendritic cell NK lectin Group Receptor-1 (DNGR-1), also known as CLEC9a, is a type II transmembrane CLR containing a single extracellular C-type lectin-like domain and a cytoplasmic tail with a hemi-immunoreceptor tyrosine-based activation motif that allows recruitment/activation of the spleen tyrosine kinase (SYK) pathway [10, 11]. DNGR1 is mostly expressed by the CD8α$^+$ subset of dendritic cells (CD8α$^+$DCs) as a receptor for necrotic cells, which favors cross-priming of cytotoxic T-lymphocytes to dead cell-associated antigens in mice [11]. Although this receptor is not essential for particle uptake via phagocytosis [11], DNGR1 is capable of internalizing bound antigens via endocytosis and directing them to the endosomal vesicles for cross-presentation of dead cell associated antigens by CD8α$^+$ DCs [12, 13].

Our hypothesis is that accumulation of apoptotic and necrotic debris during the development of atherosclerosis activates DNGR1, leading to disease progression. Here, we show that DNGR1 expression in hematopoietic cells and particularly in CD8α$^+$DCs promotes atherosclerosis through down-regulation of the anti-inflammatory and anti-atherogenic cytokine, IL10.

METHODS

Detailed methods can be found in the online Data Supplement.

Animals.

Il10$^{-/-}$, *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were from the Jackson Laboratory. C57BL/6 *Clec9a*$^{-/-}$ mice are from Reis e Sousa lab[11]. *Clec9a*$^{-/-}$ mice were backcrossed on C57Bl/6 for more than 10 generations. Littermates C57BL/6 *Clec9a*$^{-/-}$ and C57BL/6 *Clec9*$^{+/+}$ were used in all the experiments. Briefly, these mice are *Clec9a*$^{gfp/gfp}$ in which DNGR1 expression is abrogated by the insertion of a gene encoding a membrane-anchored form of green fluorescent protein (GFP) into the *Clec9a* open reading frame[11]. CD8α$^+$ DC-deficient *(Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$) mice were obtained by crossing *Irf8*$^{flox/flox}$ (Jax) with *Cd11c*$^{Cre+}$ (Jax). *Mfge8*$^{-/-}$ mice were previously described [14]. *Apoe*$^{-/-}$*Clec9a*$^{-/-}$, *Clec9a*$^{-/-}$*Il10*$^{-/-}$ and *Clec9a*$^{-/-}$*Mfge8*$^{-/-}$ mice were generated by crossing respectively *Apoe*$^{-/-}$ to *Clec9a*$^{-/-}$, *Il-10*$^{-/-}$ to *Clec9a*$^{-/-}$ and *Mfge8*$^{-/-}$ to *Clec9a*$^{-/-}$ mice. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Clec9a*$^{-/-}$ male mice on either chow diet (20 weeks) or high fat diet (HFD) (6 weeks) were used in some experiments. We subjected seven to 10-week-old *Ldlr*$^{-/-}$male mice to medullar aplasia by 9.5 gray lethal total body irradiation. We repopulated the mice with an intravenous injection of bone marrow

cells isolated from femurs and tibias of age- and sex-matched C57Bl/6J mice: *Clec9a*$^{+/+}$, *Clec9a*$^{-/-}$or *Il10*$^{-/-}$ or *Clec9a*$^{-/-}$*Il10*$^{-/-}$or *Mfge8*$^{-/-}$ or *Clec9a*$^{-/-}$*Mfge8*$^{-/-}$ mice. After 4 weeks of recovery, mice were fed with a proatherogenic diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 5, 7 or 13 weeks.

Finally, we irradiated *Ldlr*$^{-/-}$ mice and transplanted them with 2:8 mixture of either control (CD45.1) bone marrow or *Clec9a*$^{-/-}$ bone marrow mixed with bone marrow from CD8α^{+} DC-deficient (*Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$) mice. After 4 weeks of reconstitution, the mice were put on HFD diet for 7 weeks.
All mice used in these experiments were bred and housed in a specific pathogen-free barrier facility. Experiments were conducted according to the French veterinary guidelines and those formulated by the European community for experimental animal use (L358-86/609EEC).

Extent and plaque composition of atherosclerotic lesions.
Quantification of lesion size and composition was performed as previously described[15].

Flow cytometry.
Flow cytometry analysis was performed on single cell suspensions of spleen and peritoneal cells as mentioned before[16].

Cell recovery and stimulation.
Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mM 2β-mercaptoethanol and antibiotics. Splenocytes were stimulated with LPS (1 μg/ml) (Sigma) for 48 h and then supernatants were subjected to cytokine measurements. Spleen DCs, CD8α^{+}DCs, CD8α^{-}DCs, CD3^{+} and CD4^{+} T cells were purified using Miltenyi kits. To analyze cytokine production by T cell, 10^5 CD3^{+} or CD4^{+} T cells were cultured alone or co-cultured with 10^5 CD8α^{+} DCs in 96-well microplates (total volume 200 μl/well) pre-coated overnight at 4°C with anti-CD3 antibody (5 μg/ml, Pharmingen) and anti-CD28 antibody (1 μg/ml, Pharmingen) during 48 h in presence of LPS (1 μg/ml). Neutralizing anti-IL10 receptor antibody or control IgG (R&D Systems) and water-soluble **cyclodextrin**-cholesterol (Sigma) were used in some experiments. On supernatants, ELISAs were performed to measure IL10, IL17, TNFα, IL6 and IFNγ (BD) of the different conditions.

Statistical analysis.
Values are expressed as means ± s.e.m. Differences between values were examined using Student t-test. Mann Whitney is applied in the animal studies. Values were considered significant at $P \leq 0.05$.

RESULTS

DNGR1 deficiency decreases the development of atherosclerosis.

To study the role of DNGR1 in atherosclerosis, we crossed *Clec9a*$^{-/-}$ mice with atherosclerosis-susceptible *Apoe*$^{-/-}$ mice, and generated *Apoe*$^{-/-}$*Clec9a*$^{-/-}$ mice and *Apoe*$^{-/-}$*Clec9a*$^{+/+}$ littermate controls. Both groups were put on chow diet until sacrifice at 20 weeks of age. As shown in Figure 1, invalidation of DNGR1 led to a marked 60 % decrease of plaque size (p=0.002), despite no significant change in plasma cholesterol levels (Figure 1A-C). We also reconstituted lethally-irradiated *Ldlr*$^{-/-}$ mice with *Clec9a*$^{+/+}$ or *Clec9a*$^{-/-}$ bone marrow. After 4 weeks of recovery, mice were put on HFD for 5 or 7 weeks to study early disease stages, and for 13 weeks to study advanced stages of atherosclerosis. As expected, plasma cholesterol levels significantly increased overtime during HFD, without any significant differences between mice transplanted with *Clec9a*$^{-/-}$ or *Clec9a*$^{+/+}$ bone marrow cells (Supplementary Fig. IA). Mice reconstituted with *Clec9a*$^{-/-}$ bone marrow showed significant reduction in atherosclerosis at the aortic sinus (Figure 1D and E) compared with mice reconstituted with *Clec9a*$^{+/+}$ bone marrow after 5 or 7 weeks of

HFD, confirming the atheroprotective effect of DNGR1 deletion. Plaque area was also significantly decreased in the thoracic aorta after 7 weeks of HFD in mice reconstituted with *Clec9a* $^{-/-}$ compared to *Clec9a* $^{+/+}$ bone marrow (Figure 1F). However, DNGR1 deletion in bone marrow-derived cells had no effect on atherosclerosis in mice fed HFD for 13 weeks (Figure 1D and E). Also, there was no difference in plaque size between *Apoe*$^{-/-}$*Clec9a*$^{+/+}$ and *Apoe*$^{-/-}$*Clec9a*$^{-/-}$ mice fed a HFD during 6 weeks (Supplementary Fig. IB and C).

This was a surprising finding given that necrotic cores, which may activate signaling by DNGR1, show increased accumulation in lesions at the advanced stages of atherosclerosis (Supplementary Fig.ID). To account for that finding, we hypothesized that the absence of DNGR1 pro-atherogenic effects after 13 weeks of HFD in *Ldlr*$^{-/-}$ mice or after HFD in *Apoe*$^{-/-}$ mice was due to the very high plasma cholesterol levels observed in these mice, rather than to the advanced stage of the plaques.

We then examined *Clec9a mRNA* in the spleen, a tissue known to readily express Clec9a [17] and to play a major role in atherosclerosis [18 19]. As shown in Supplementary Figure IIA, Clec9a chimerism was high at all time points (7 or 13 weeks), as assessed by the almost total absence of Clec9a expression in spleens of mice reconstituted with DNGR1-deficient bone marrow. However, Clec9a expression in spleens of *Ldlr*$^{-/-}$ mice reconstituted with *Clec9a*$^{+/+}$ bone marrow markedly decreased after 13 weeks of HFD, compared with that of *Ldlr*$^{-/-}$ mice on chow diet or after 7 weeks of HFD (Supplementary Fig.IIA). This may suggest that *Clec9a* expression was down regulated by the prolonged and severe hypercholesterolemia after 13 weeks of HFD. Consistent with this hypothesis, in vitro incubation of splenocytes from *Clec9a*$^{Gfp/Gfp}$ mice, in which the *Clec9a* gene was replaced by a GFP reporter gene (see Materials and Methods) in the presence of cholesterol markedly decreased GFP expression (Supplementary Fig. IIB).

In order to investigate the role of Clec9a in advanced atherosclerosis whilst avoiding severe and prolonged hypercholesterolemia, we generated *Clec9a*$^{-/-}$*Mfge8*$^{-/-}$ mice by crossing *Mfge8*$^{-/-}$ mice, in which the accumulation of late apoptotic/necrotic cells is increased due to defective efferocytosis [20, 21], with *Clec9a* $^{-/-}$ mice. Then, lethally-irradiated male *Ldlr*$^{-/-}$ mice were reconstituted with either *Mfge8*$^{-/-}$ or *Clec9a*$^{-/-}$*Mfge8*$^{-/-}$ bone marrow cells, and put on HFD for 7 weeks. As expected, a very small apoptotic area as assessed by TUNEL staining was detected in plaques of *Ldlr*$^{-/-}$mice reconstituted with *Clec9a* $^{+/+}$ or *Clec9a* $^{-/-}$ bone marrow at this early stage of disease development (Supplementary Fig. IIIA-B). As previously published [9], Mfge8 deficiency markedly increased apoptotic TUNEL^{+} area (Supplementary Fig. IIIA-B), and accelerated lesion development (Supplementary Fig. IIIC-D) under 'moderate' hypercholerolemia (5.01±0.66 g/L *vs* 5.86±0.45 g/L, p=0.33 in WT and *Mfge8*$^{-/-}$ mice, respectively). No significant changes of necrotic core area was observed between *Clec9a* $^{-/-}$*Mfge8*$^{-/-}$ and *Mfge8*$^{-/-}$ groups (data not shown). Interestingly, atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice reconstituted with *Mfge8* $^{-/-}$ bone marrow at 7 weeks of HFD was similar to that of *Ldlr*$^{-/-}$mice fed a HFD for 13 weeks. Remarkably, *Ldlr*$^{-/-}$ mice transplanted with *Clec9a* $^{-/-}$*Mfge8*$^{-/-}$ bone marrow showed a marked reduction of atherosclerosis (Supplementary Fig. IIIC-D) as compared with mice transplanted with *Mfge8*$^{-/-}$ bone marrow, despite no change in plasma cholesterol levels (5.86±0.45 g/L *vs* 6.19±0.33 g/L, p=0.56 in *Mfge8*$^{-/-}$ and *Clec9a* $^{-/-}$*Mfge8*$^{-/-}$ mice, respectively). This indicates that DNGR1 promotes atherosclerosis even at advanced stages of lesion development, but only in a context of 'moderate' hypercholesterolemia.

DNGR1 mediates pro-atherogenic effects through down-regulation of IL10.

We next assessed lesion composition in *Clec9a* $^{-/-}$ → *Ldlr*$^{-/-}$ and *Clec9a* $^{+/+}$ → *Ldlr*$^{-/-}$ mice after 5, 7 and 13 weeks of HFD. The percentage of macrophages (MOMA-2 staining/plaque surface) showed a significant decrease in mice reconstituted with DNGR1-deficient bone marrow after 5, 7 or 13 weeks of HFD, compared with chimeric *Clec9a* $^{+/+}$ mice (Supplementary Fig. IVA). T lymphocyte accumulation within the lesions (CD3 staining) significantly decreased in mice transplanted with *Clec9a* $^{-/-}$, compared to

Clec9a $^{+/+}$ at 7 weeks of HFD (p=0.047) (Figure 2A), but this effect was lost at 13 wks of HFD (p= 0.77)(Figure 2B) when plasma cholesterol levels were excessively high.

We then examined the inflammatory profile in spleens of *Ldlr*$^{-/-}$mice reconstituted with either *Clec9a* $^{+/+}$or *Clec9a* $^{-/-}$ bone marrow. The expression of major inflammatory cytokines involved in atherosclerosis, including *Ifnγ*, *Il1β* and *Il6* [22] was not different between the 2 groups of mice after 7 weeks of HFD (Supplementary Fig. IVB). However, as shown in Supplementary Fig. VA-B and Figure 2C, DNGR1 deficiency after 5 and 7 weeks, but not after 13 weeks of HFD, markedly increased *Tgfβ* and *Il10* expression, suggesting an anti-inflammatory and anti-atherogenic phenotype [23-26]. We also found a significant increase of IL10 production by splenocytes in the absence of DNGR1 after lipopolysaccharide (LPS)-TLR4 stimulation (Supplementary Figure VC), without significant changes in TNFα or IL6 (data not shown), suggesting that DNGR1 acts as a regulator of IL10.

To directly address the physiological relevance of enhanced expression of IL10 by DNGR1-deficient bone marrow cells, we generated *Clec9a* $^{-/-}$*Il10*$^{-/-}$ mice. We recovered bone marrow from *Il10*$^{-/-}$or *Clec9a* $^{-/-}$*Il10*$^{-/-}$ mice to reconstitute lethally-irradiated *Ldlr*$^{-/-}$mice. After 4 weeks of recovery, mice were put on HFD for 7 weeks. As previously reported [27], IL10 deficiency in the bone marrow accelerated lesion development (Figure 3A-B), despite no change in plasma cholesterol levels (5.00 ±0.78 g/L *vs* 4.57±0.20 g/L, p=0.93). Strikingly, the observed atheroprotective effect of *Clec9a* deficiency (see Figure 1D) was completely abolished in *Clec9a* $^{-/-}$*Il10*$^{-/-}$ → *Ldlr*$^{-/-}$ mice, (Figure 3A-B), despite no impact on plasma cholesterol levels (4.57±0.20 g/L *vs* 4.81±0.20 g/L, p=0.53). Clec9a expression in *Il10*$^{-/-}$ → *Ldlr*$^{-/-}$ spleens is comparable to *Il10*$^{+/+}$ → *Ldlr*$^{-/-}$ indicating that inflammation is not involved in the modulation of Clec9a expression (data not shown). Moreover, no significant differences in necrotic core area, macrophage or T cell infiltration were observed between *Ldlr*$^{-/-}$mice reconstituted with bone marrow cells from either *Il10*$^{-/-}$ *or Clec9a* $^{-/-}$ *Il10*$^{-/-}$ (Figure 3C-E). This data further demonstrate that the modulation of IL10 expression is an important mechanism through which DNGR1 mediates its pro-atherogenic effects.

DNGR1 expressed in CD8α^{+} *DCs regulates IL10 production and atherosclerosis development.*

DNGR1 is highly expressed in CD8α^{+} DCs [11]. However, the role of CD8α^{+} DCs in atherosclerosis is still poorly understood [28, 29]. To address the direct role of CD8α^{+} DC-specific DNGR1 in atherosclerosis, we used irradiated *Ldlr*$^{-/-}$ mice transplanted with a 8:2 mixture of CD8α^{+} DC-deficient *(Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$) (**S**upplementary Figure VIA) CD45.2 bone marrow (80%), mixed with either *Clec9a* $^{+/+}$ (CD45.1) or *Clec9a* $^{-/-}$ bone marrow (20%). In these chimeric mice, at least 80% of spleen cells other than CD8α^{+} DCs, including T cells (CD4^{+} and CD8^{+}), B cells (B220^{+}), CD8α^{-} DCs, macrophages (F4/80^{+}) and neutrophils (GR1^{+}), derived from the 80% *Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$ (CD45.2) bone marrow (Supplementary Figure VIB and Figure 4A). On the other hand, we expected all CD8α^{+} DCs to reconstitute almost exclusively from the 20% bone marrow (*Clec9a* $^{+/+}$ or *Clec9a* $^{-/-}$). In agreement with this, *Clec9a* mRNA was highly expressed in purified spleen CD8α^{+} DCs of mice reconstituted with 80% *Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$ + 20% *Clec9a* $^{+/+}$ bone marrow (CD8α^{+} DC-Cont), but markedly decreased in purified spleen CD8α^{+} DCs of mice reconstituted with 80% *Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$ + 20% *Clec9a* $^{-/-}$ bone marrow (CD8α^{+} DC- *Clec9a* $^{-/-}$) (Figure 4B). As expected, DNGR1 was barely expressed in purified CD8α^{-} DCs from spleens of both groups of mice (Figure 4B). Reconstitution of spleen DCs, and more particularly CD8α^{+} DCs, was similar between the 2 groups of mice (Supplementary Figure VIC).

Remarkably, the selective absence of DNGR1 in CD8α^{+} DCs significantly decreased plaque size (p=0.04) (Figure 4C and D), despite no change in plasma cholesterol levels (6.69±0.25 g/L *vs* 6.44±0.31 g/L, p=0.46). Moreover, macrophage and T cell infiltration within the plaques were significantly decreased in the absence of DNGR1 in CD8α^{+} DCs, indicating that the expression of DNGR1 in CD8α^{+} DCs is required to mediate its pro-atherogenic effects (Supplementary Figure VII A and Figure 5A and B).

DNGR1 has previously been shown to induce $CD8^+$ T-cell responses against dying cells [30]. However, we found no differences in spleen $CD8^+$ T-cell percentage or activation between the 2 groups of mice (Supplementary Fig. VII B), suggesting the involvement of other mechanisms.

Interestingly, as in the total DNGR1-deficiency model, the specific deletion of DNGR1 in $CD8\alpha^+$ DCs led to a marked increase of IL10 expression in those cells (Supplementary Fig.VIIC) but also more generally in the spleen (Figure 5C), showing that DNGR1 specifically expressed by $CD8\alpha^+$ DCs plays a major role in the regulation of IL10. DNGR1 was also detected in $CD45^+$ cells purified from atherosclerotic plaque aorta (Supplementary Fig. VIIIA), suggesting potential local effects. We also found higher *Il10* mRNA levels in aortas of mice with DNGR1 deletion in $CD8\alpha^+$ DCs compared to controls, without significant changes of *Tnfα*, *Il6* and *Tgfβ* expression (Supplementary Fig. VIIIB).

The expression of *Il10* in $CD8\alpha^+$ DCs and DCs in general was relatively weak, which suggests that DNGR1 deficiency in $CD8\alpha^+$ DCs likely increased IL10 expression in other cell types. Accordingly, it was previously shown that IL10, and particularly DC-derived IL10 promoted IL10-producing T regulatory (Tr1) cells [31, 32]. In this regard, we found that co-culture of splenic $CD8\alpha^+$ DCs purified from $Il10^{-/-}$ mice with $Il10^{+/+}$ $CD3^+$ T cells resulted in a significantly lower IL10 production as compared with the co-culture of $Il10^{+/+}$ $CD8\alpha^+$ DCs with $Il10^{+/+}$ $CD3^+$ T cells, further highlighting the importance of $CD8\alpha^+$ DC-derived IL-10 to stimulate IL10 production by T cells (Supplementary Figure IXA). Furthermore, co-culture of DNGR1-deficient $CD8\alpha^+$ DCs with $CD3^+$ T cells significantly increased IL10 production by T cells without significant changes of IFNγ or IL17 (Supplementary Figure IXB-D). In line with these results, the selective absence of DNGR1 in $CD8\alpha^+$ DCs significantly increased IL10 expression in T-$CD4^+$ cells in vivo (Figure 5D), suggesting a specific bystander effect of DNGR1 expressed in $CD8\alpha^+$ DCs on the regulation of IL10 production by $CD4^+$ cells. Of note, DNGR1 in CD8α+DCs did not affect the levels of T regulatory cells ($CD4^+CD25^+FOXP3^+$) (Supplementary Figure XA).

To directly test the specific role of IL10 expression in $CD8\alpha^+$ DCs in the presence or absence of DNGR1, we irradiated and transplanted $Ldlr^{-/-}$ mice with 8:2 bone marrow from $Cd11c^{Cre+}/Irf8^{flox/flox}$ (80%) mice, mixed with 20% of bone marrow from either control (n=8), $Clec9a^{-/-}$ (n=9) $Il10^{-/-}$ (n=8) or $Clec9a^{-/-}$ $Il10^{-/-}$ (n=9) mice. Then, the mice were put on HFD during 7 weeks. As shown in Figure 6A-D, specific IL10 deletion in $CD8\alpha^+$ DC abrogated the atheroprotection observed in absence of DNGR1 in this cell type, without any significant change of plasma cholesterol levels. This was associated with the inability of Clec9a deficiency to increase IL10 production by splenocytes (Figure 6E). Furthermore, co-culture of $CD8\alpha^+$ DCs from each group with wild-type $CD4^+$ T cells showed that $CD8\alpha^+$ DCs promoted increased IL10 production by T cells in the absence of DNGR-1, which was dependent on IL10 expression in $CD8\alpha^+$ DC (Supplementary Figure XB), and most likely IL10 receptor signaling in $CD4^+$ T cells (Supplementary Figure XC).

DISCUSSION

Accumulation of apoptotic cells or debris during the development of atherosclerosis promotes inflammation, and contributes to plaque growth and thrombosis upon plaque rupture [5, 33]. We recently showed that C-type lectin receptor 4e (CLEC4e), known to be involved in the sensing of necrotic cores, promoted atherosclerosis through induction of endoplasmic reticulum stress-dependent macrophage proliferation, inflammation, and foam cell formation[34]. However, other C-type lectin receptors could recognize necrotic cores, and stimulate inflammation and plaque progression.

Our findings provide *in vivo* and *in vitro* evidence that DNGR1, known to be involved in the sensing and presentation of antigens derived from necrotic cells[12], is pro-atherogenic, and its deletion in both *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mouse models promotes an anti-inflammatory response and deactivates important pro-atherogenic mechanisms. We have identified a new DNGR1-regulated IL10 pathway that controls vascular inflammation, and substantially impacts lesion development. DNGR1 deficiency inhibited macrophage and T cell infiltration within the plaques and up-regulated *Tgfb* and *Il10*, two major anti-inflammatory and anti-atherogenic genes [2, 25]. Intriguingly, the atheroprotective effect of DNGR1 deletion was already observed at the early stages of atherosclerosis (5 or 7 weeks of HFD) when plaques were still relatively small and apoptotic cell death was barely detectable. Even though we cannot rule out the possibility that DNGR1 was activated by some necrotic debris, it is likely that other PRRs, including TLRs, were activated during atherosclerosis [35] and interacted with DNGR1 signaling to activate inflammatory pathways. In agreement with this, our results showed that the absence of DNGR1 in splenocytes increased the production of the anti-inflammatory factor IL10 following TLR4 stimulation.

After 13 weeks of HFD, we no longer observed differences in plaque size in the absence of DNGR1, despite the presence of necrotic cores. Also, the atheroprotection observed in absence of DNGR1 in *Apoe*$^{-/-}$ mice fed with chow diet was abrogated when the mice were put on HFD. We believe that this was due to reduced Clec9a expression caused by the very high plasma cholesterol levels, as indicated by our in vitro experiments showing reduced CLEC9a expression by CD8α+DCs in the presence of high cholesterol. In agreement with this, we found that DNGR1 deletion was still atheroprotective in mice deficient for *Mfge8*, which exhibited after only 7 weeks of HFD (moderate hypercholesterolemia) increased accumulation of apoptotic/necrotic debris, and plaques as large as those found in *Mfge8*$^{+/+}$ mice after 13 weeks of HFD (severe hypercholesterolemia). In addition, previous studies showed that T cells become dispensable for atherosclerosis development after prolonged and severe hypercholesterolemia [36, 37]. More recently, Tsiaoui et al. reported that when *Apoe*$^{-/-}$ mice were fed a chow diet for 35 weeks, lesions were approximately the same size as after 12 weeks of HFD. Yet, T-bet deficient *Apoe*$^{-/-}$ mice, in which T cells are unable to differentiate into a Th1 phenotype, exhibited fewer plaques compared with immunocompetent *Apoe*$^{-/-}$ mice after 35 weeks of chow diet, while T-bet deficiency had no athero-protective effects in *Apoe*$^{-/-}$ mice fed a HFD for 12 weeks [38]. As our data suggest that the protective effects of DNGR1 deletion are dependent on T cells, this may explain why the athero-protective effect is lost at very high plasma cholesterol levels.

In agreement with our hypothesis of a role for DNGR1 in adaptive immune responses, earlier studies showed a restricted DNGR1 expression in CD8α$^+$ DCs [12, 39]. However, existing reports about the role of CD8α$^+$ DCs in atherosclerosis are contradictory. One study used FMS-like tyrosine kinase (*flt3*)$^{-/-}$ *Ldlr*$^{-/-}$ double deficient mice, which displayed marked depletion of CD8α$^+$ DCs, suggested a pro-atherogenic role of CD8α$^+$ DCs [28]. However, *flt3*$^{-/-}$ mice are also deficient for many other conventional DC subtypes. Moreover, in contrast to those results, atherosclerotic plaque formation and stability were not altered in chimeric *Ldlr*$^{-/-}$ mice transplanted with bone marrow from Basic leucine zipper transcription factor ATF-like 3 (*Batf3*)-deficient mice that lack the CD8α$^+$ DC subset, compared to *Ldlr*$^{-/-}$ mice transplanted with a control bone marrow [29]. Our study differs from the previous ones in that we report on the deletion of a specific PRR selectively expressed by CD8α$^+$ DCs, while preserving the other functions of this DC subset. Our present results clearly indicate that the specific deletion of DNGR1 in CD8α$^+$ DCs promotes

atherosclerosis, identifying a previously unrecognized pro-atherogenic function of CD8α$^+$ DCs in the development of atherosclerosis.

DNGR1 stimulation in CD8α$^+$ DCs has been previously shown to mediate cross-presentation of dead-cell-associated antigens leading to CD8$^+$ T cell activation [11, 40]. Thus, we expected that DNGR1 expressed in CD8α$^+$ DCs under conditions of hyperlipidemia would lead to CD8$^+$ T cell activation. However, our results showed no significant changes in spleen CD8$^+$ T cell count or activation between control and DNGR1-deficient mice, suggesting that cross-presentation may not be the main mechanism accounting for DNGR1-mediated pro-atherogenic effects. In addition, the role of CD8$^+$ T cells and cross-presentation in atherosclerosis is still debated [29, 41-43]. Interestingly, we found that DNGR1 deletion in CD8α$^+$ DCs increased the expression of the anti-inflammatory and anti-atherogenic IL10 in spleens, suggesting the involvement of this cytokine in the observed athero-protective effects. Noteworthy, spleen CD8α$^+$ DCs purified from mice reconstituted with DNGR1 deficient CD8α$^+$ DCs showed a significantly higher IL10 expression compared with control CD8α$^+$ DCs. Yet, IL10 expression by CD8α$^+$ DCs was weak, suggesting an additional impact of DNGR1 deficiency in CD8α$^+$ DCs on IL10 expression in other cell types. In agreement with this, we found that the absence of DNGR1 in CD8α$^+$ DCs led to increased IL10 (but not IL17 nor IFNγ) production by CD4$^+$ T cells, suggesting the induction of an anti-inflammatory and anti-atherogenic T cell response.

Our findings point to an important role of IL10 as the main atheroprotective factor in the absence of DNGR1. First, IL10 expression was up-regulated at 5 and 7 weeks of high fat diet in the absence of DNGR1. However, after 13 weeks of high diet, when no difference of plaque size was observed, IL10 expression remained unchanged in the absence of DNGR1. Moreover, double deficiency in DNGR1 and IL10, specifically in CD8α$^+$ DCs completely abrogated the protective effects of DNGR1 deletion. The mechanisms leading to an increase of IL10 in the absence of DNGR1 are still unknown and will require further investigation. One likely mechanism is that interaction between TLRs and DNGR1 signaling modulates IL10 production through the SYK pathway [10, 11, 44, 45].

In conclusion, our results identify a pro-atherogenic role for the necrotic cell sensor DNGR1 through the regulation of IL10 production, and suggest a new potential therapeutic target to combat atherosclerosis.

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DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1: DNGR1 deletion in bone marrow protects against atherosclerosis A) Plasma cholesterol levels in 20 week-old male *Apoe*$^{-/-}$ (n=10) and male *Apoe*$^{-/-}$ *Clec9a* $^{-/-}$(n=13) put on chow diet during 20 weeks. **B-C)** Representative photomicrographs and quantification of lesion size in aortic sinus of male *Apoe*$^{-/-}$ (n=10) and male *Apoe*$^{-/-}$ *Clec9a* $^{-/-}$ mice (n=13) **(D-E)** Representative photomicrographs and quantification of lesion size in *Ldlr*$^{-/-}$ mice reconstituted with either control (*Clec9a* $^{+/+}$ → *Ldlr*$^{-/-}$) or *Clec9a* $^{-/-}$ (*Clec9a* $^{-/-}$ → *Ldlr*$^{-/-}$) bone marrow after 5 weeks (control (n=7) and *Clec9a* $^{-/-}$ (n=8)) or 7 weeks (control (n=12) and *Clec9a* $^{-/-}$ (n=16)) or 13 weeks (control (n=8) and *Clec9a* $^{-/-}$ (n=9)) of high fat diet (HFD)**. F)** Representative photomicrographs and quantification of lesion size in the thoracic aorta of reconstituted with either control (*Clec9a* $^{+/+}$ → *Ldlr*$^{-/-}$ (n=12)) or *Clec9a* $^{-/-}$ (*Clec9a* $^{-/-}$ → *Ldlr*$^{-/-}$ (n=16)) bone marrow after 7 weeks of HFD. Mean values ± SEM are shown. * P<0.05, **P<0.001, ***P<0.0001

Figure 2: DNGR1 deletion protects against vascular inflammation. A-B) Representative photomicrographs and quantitative analysis of lesional T cell infiltration in *Ldlr*$^{-/-}$ mice reconstituted with either Control (n=12) and *Clec9a* $^{-/-}$ (n=16) bone marrow after 7 weeks or Control (n=8) and *Clec9a* $^{-/-}$ (n=9) after 13 weeks of high fat diet (HFD). Plaque (P) and Media (M) are indicated. The results showed a significant decrease of T cells % (CD3 area: plaque area) in absence of DNGR1 after 7 weeks but not after 13 weeks of HFD. **C**) *Clec9a*, *Tgf-β* and *Il-10 mRNA* in spleens of *Ldlr*$^{-/-}$ mice transplanted with Cont (n=7) or *Clec9a* $^{-/-}$ (n=9) bone marrow after 7 weeks of HFD. The results show a significant increase of Tgf- β and Il-10 expression in absence of DNGR1. Mean values ± SEM are shown. *P<0.05, **P<0.001, ***P<0.0001

Figure 3: Il10 deficiency abrogates athero-protection in DNGR1-deficient mice. A-B) Representative photomicrographs and quantification of lesion size in the aortic sinus of lethally-irradiated male *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from *Clec9a*$^{+/+}$ (n=4) or *Clec9a*$^{-/-}$ (n=5) or *Il10*$^{-/-}$ (n=6) or *Clec9a* $^{-/-}$*Il10*$^{-/-}$ (n=7) mice after 7 weeks of high fat diet (HFD). **C-E**) Representative photomicrographs and quantitative analysis of necrotic cores, lesional macrophage and T cell infiltration in *Ldlr*$^{-/-}$ mice reconstituted with either *Il10*$^{-/-}$ (n=6) and *Clec9a* $^{-/-}$*Il10*$^{-/-}$ (n=7) bone marrow after 7 weeks of HFD. The results show the importance of Il-10 in DNGR1-mediated pro-atherogenic effects. Results were obtained after 4 weeks of recovery from bone marrow transplantation followed by 7 weeks on a HFD. Mean values ± SEM are shown. *P<0.05, **P<0.001, ***P<0.0001.

Figure 4: DNGR1 deletion in CD8α^{+}DCs protects against atherosclerosis. A) Representative example and quantitative analysis of flow cytometry-donor origin defined as CD45.1 % and CD45.2 % of spleen cells from chimeric *Ldlr*$^{-/-}$ mice transplanted with bone marrow mix (2:8) from control (CD45.1) (n=7) or *Clec9a* $^{-/-}$ (n=8) bone marrow cells mixed with *Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$ (CD45.2) bone marrow. After bone marrow reconstitution, the mice were put on a HFD during 7 weeks. The results show that the majority of spleen cells (80%) originates from CD45.2 bone marrow**. B)** *Clec9a* mRNA in pooled purified CD8α^{+}DCs isolated from either control group (CD8α^{+}DC-Cont, n=7) or DNGR1-deficient CD8α^{+}DC group (CD8α^{+}DC-*Clec9a* $^{-/-}$, n=8). (**C-D**) Representative photomicrographs and quantification of lesion size in aortic sinus of chimeric *Ldlr*$^{-/-}$ mice transplanted with 2:8 mix of bone marrow from control (CD45.1) (n=7) or *Clec9a* $^{-/-}$ (n=8) bone marrow cells mixed with *Cd11c*$^{Cre+}$*/Irf8*$^{flox/flox}$ (CD45.2) bone marrow. The results show that the deletion of DNGR1 in CD8α^{+}DCs protects against atherosclerosis. Mean values ± SEM are shown. P<0.05, **P<0.001, ***P<0.0001.

Figure 5: DNGR1 deletion in CD8α^{+}DCs alleviates inflammation in atherosclerosis. A-B) photomicrographs and quantitative analysis of lesional T cell infiltration in *Ldlr*$^{-/-}$ mice reconstituted with either control group (CD8α^{+}DC-Cont, n=7) or DNGR1-deficient CD8α^{+}DC group (CD8α^{+}DC-Clec9a $^{-/-}$, n=8) after 7 weeks of HFD. Plaque (P) and media (M) are indicated. **C)** *Il-10 mRNA* in spleens of control

group (CD8α$^+$DC-Cont, n=7) or DNGR1-deficient CD8α$^+$DC group (CD8α$^+$DC-Clec9a $^{-/-}$, n=8). **D)** Representative examples and quantitative analysis of flow cytometry-based intracellular staining of IL-10 gated on CD4+ cells from the DNGR1-deficient CD8α$^+$DCs (n=8) compared to control-CD8α$^+$DCs (n=7) groups of mice. The results show the importance of DNGR1 in CD8α$^+$DC-mediated pro-atherogenic effects. Mean values ± SEM are shown. *P<0.05, **P<0.001, ***P<0.0001.

Figure 6: IL-10 deletion abrogates protective effects of DNGR-1 absence in CD8α$^+$DCs. A-B) Representative photomicrographs and quantification of lesion size in aortic sinus **C)** plasma cholesterol in chimeric *Ldlr*$^{-/-}$ mice transplanted with 2:8 mix of bone marrow from control (n=8) or *Clec9a* $^{-/-}$ (n=9) or *Il-10*$^{-/-}$ (n=8) or *Clec9a*$^{-/-}$*Il-10*$^{-/-}$ (n=9) bone marrow cells mixed with *Cd11c*$^{Cre+}$/*Irf8*$^{flox/flox}$ bone marrow after 7 wks of high fat diet (HFD).**D)** *Clec9a and Il-10* mRNA in pooled purified CD8α$^+$DCs isolated from either control group (CD8α$^+$DC-Cont, n=8) or DNGR1-deficient CD8α$^+$DC group (CD8α$^+$DC-*Clec9a* $^{-/-}$, n=9) or IL-10-deficient CD8α$^+$DC group (CD8α$^+$DC-*Il-10* $^{-/-}$, n=8) or DNGR-1/IL-10-deficient CD8α$^+$DC group (CD8α$^+$DC-*Clec9a*$^{-/-}$*Il-10* $^{-/-}$, n=9). **E)** IL-10 production by anti-CD3 (5ug/ml) and anti-CD28 (1 ug/ml) stimulated splenocytes from either CD8α$^+$DC-Cont (n=8) or CD8α$^+$DC-*Clec9a* $^{-/-}$ (n=9) or CD8α$^+$DC-*Il-10* $^{-/-}$ (n=8) or CD8α$^+$DC-*Clec9a*$^{-/-}$*Il-10*$^{-/-}$ (n=9), in presence of LPS (1μg/ml). The results show that the deletion of IL-10 abrogates the protective effects of DNGR1 deletion in CD8α$^+$DCs. Mean values ± SEM are shown. P<0.05, **P<0.001, ***P<0.0001

NOVELTY AND SIGNIFICANCE

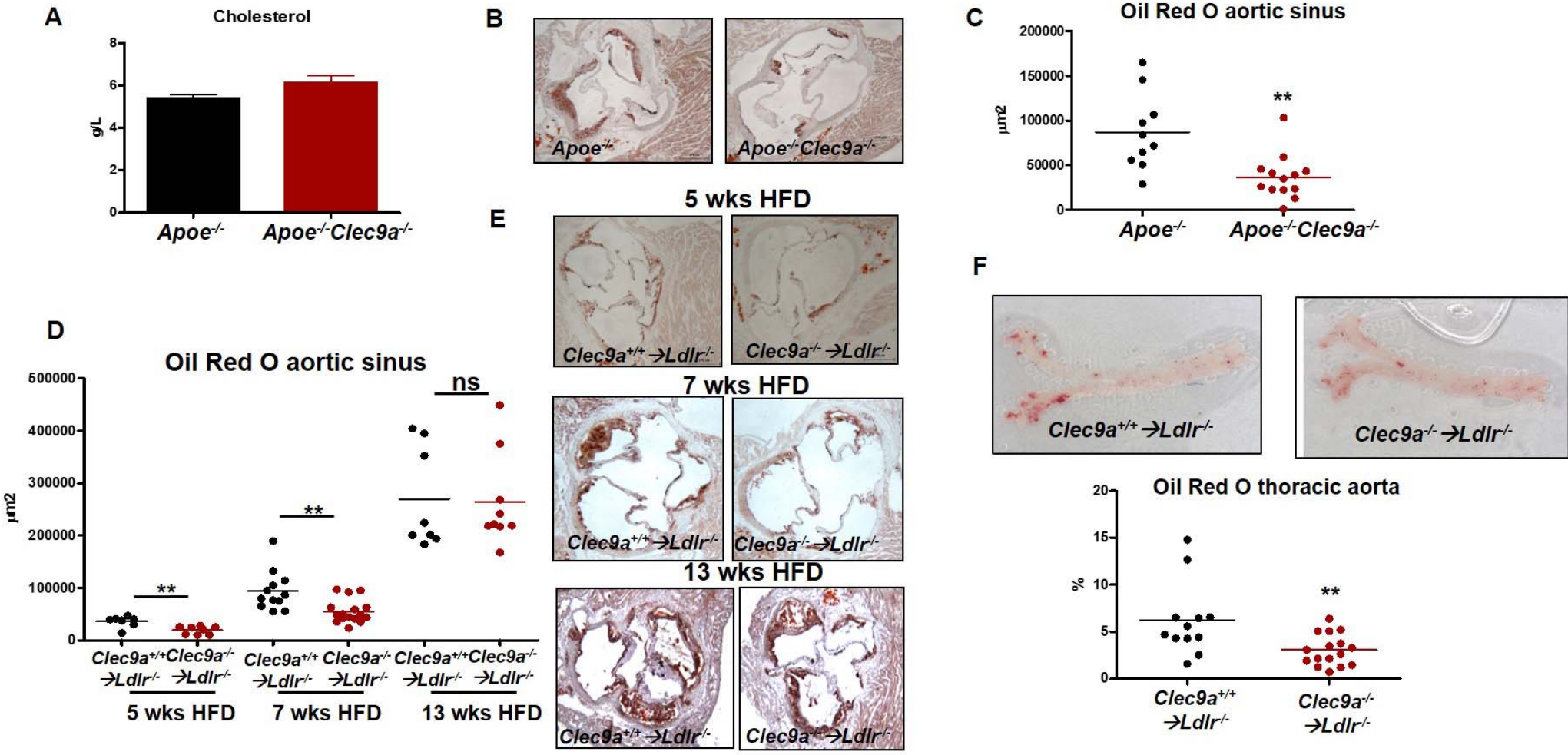
What Is Known?

- Advanced atherosclerotic plaques are characterized by an increase i necrotic cells.
- A necrotic core increases plaque instability.
- The DNGR1 (Dendritic cell NK lectin Group Receptor-1) is involved in sensing necrotic cells.

What New Information Does This Article Contribute?

- The DNGR1, specifically expressed by a subtype of dendritic cells ($CD8\alpha^+$DCs), has pro-inflammatory and pro-atherogenic effects that depend on interleukin 10 (IL-10).
- A new role of DNGR1 in regulating vascular inflammation and atherosclerosis, making it a potential target that could be blocked to promote plaque stabilization.

Accumulation of necrotic debris increases inflammation in atherosclerotic lesions and has been associated with higher susceptibility to plaque rupture. However, the mechanisms that contribute to necrosis-induced inflammation during atherogenesis remain unclear. DNGR1 is a sensor of necrotic cells that is preferentially expressed in a subtype of dendritic cells ($CD8\alpha^+$DCs). We have shown that in a context of moderate hypercholesterolemia the total bone marrow and $CD8\alpha^+$DC-specific DNGR1 deletion decreased the infiltration of T lymphocytes and macrophages within lesions as well as plaque size. Mechanistically, the absence of DNGR1 led to increased expression of Il-10 in $CD8\alpha^+$DCs, which also appeared to impact the production of IL-10 by CD4+ cells. Moreover, deletion of Il-10 in $CD8\alpha^+$DCs abolished the atheroprotective effects of DNGR1 deficiency. Further studies are required to determine whether increased DNGR1 is associated with the formation of thin-cap fibroatheromatous lesions in humans and whether blockage of this protein would promote plaque stabilization.


A
Cholesterol
g/L
Apoe-/-
Apoe-/-Clec9a-/-
B
Apoe-/-
Apoe-/-Clec9a-/-
C
Oil Red O aortic sinus
µm2
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Apoe-/-
Apoe-/-Clec9a-/-
D
Oil Red O aortic sinus
µm2
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ns
Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
5 wks HFD
7 wks HFD
13 wks HFD
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5 wks HFD
Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
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Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
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Clec9a+/+ →Ldlr-/-
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Oil Red O thoracic aorta
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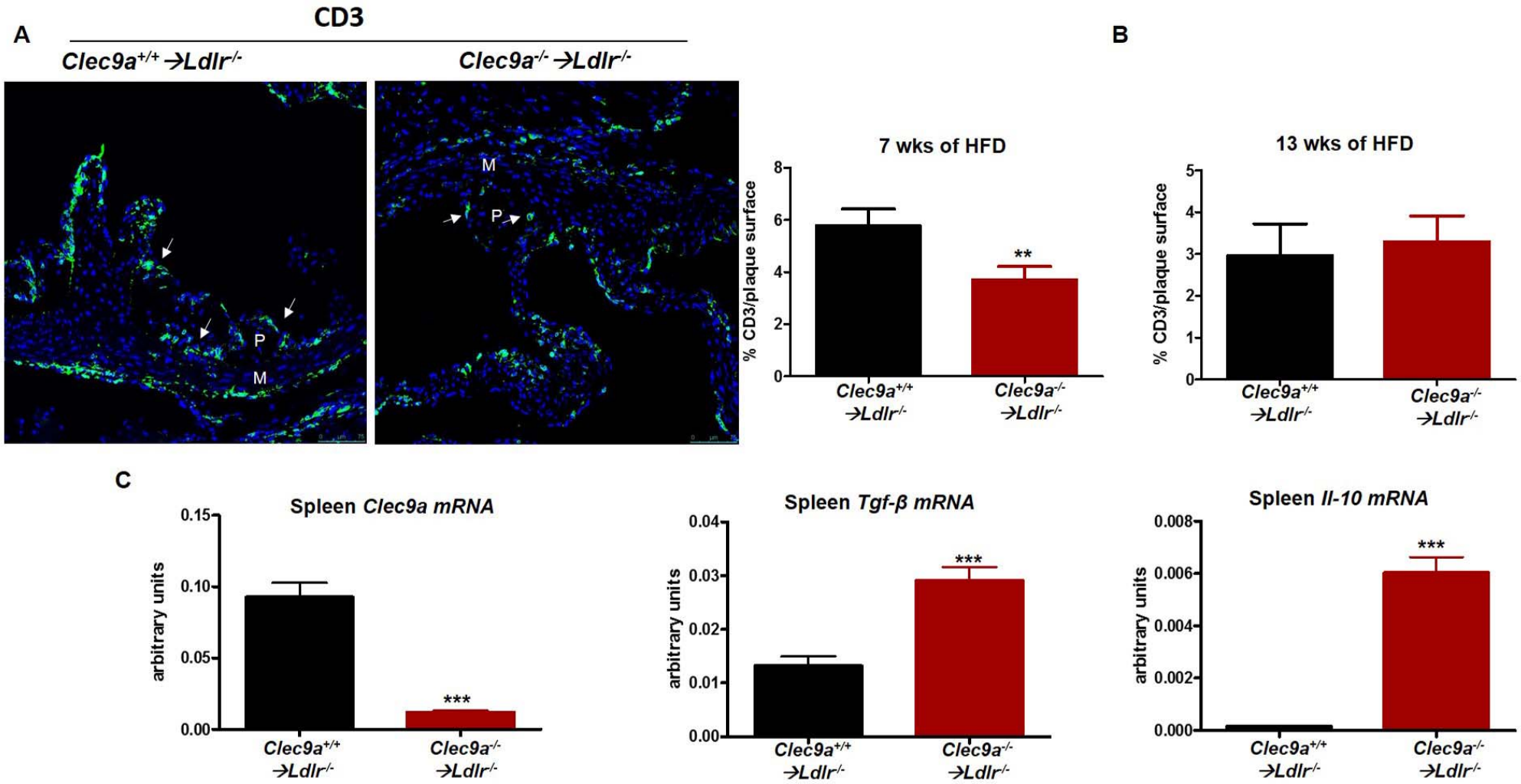
Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-

Figure 1


A
CD3
Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
M
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P
B
7 wks of HFD
% CD3/plaque surface
0
2
4
6
8
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Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
13 wks of HFD
% CD3/plaque surface
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Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
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Spleen Clec9a mRNA
arbitrary units
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Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
Spleen Tgf-β mRNA
arbitrary units
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0.01
0.02
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Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-
Spleen Il-10 mRNA
arbitrary units
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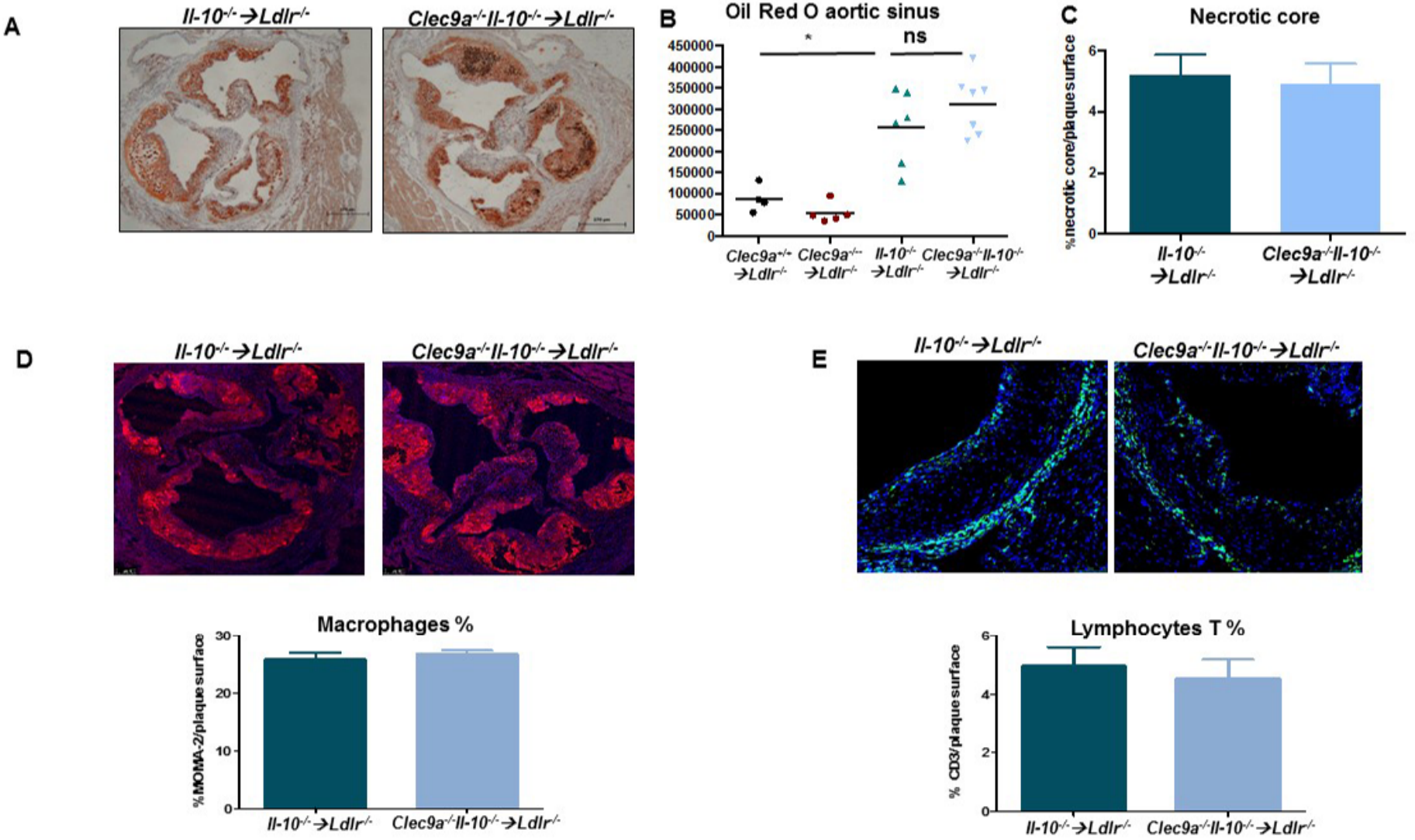
Clec9a+/+ →Ldlr-/-
Clec9a-/- →Ldlr-/-

Figure 2


A
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-
B
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350000
300000
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200000
150000
100000
50000
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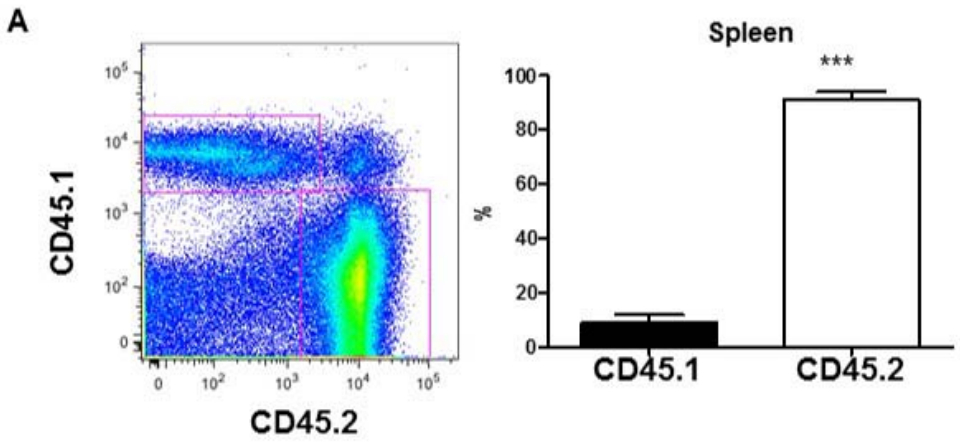
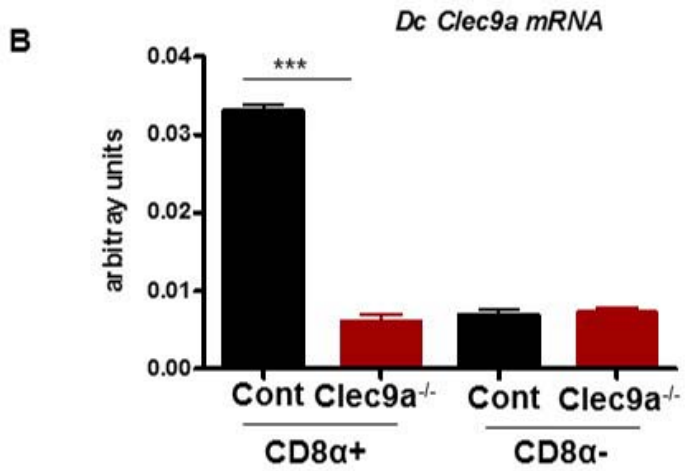
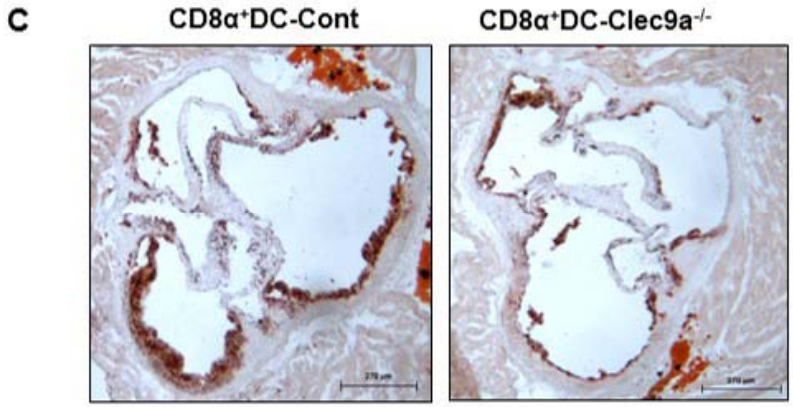
Clec9a+/-→Ldlr-/-
Clec9a-/-→Ldlr-/-
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-
C
Necrotic core
% necrotic core/plaque surface
6
4
2
0
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-
D
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-
Macrophages %
% MOMA-2/plaque surface
30
20
10
0
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-
E
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-
Lymphocytes T %
% CD3/plaque surface
6
4
2
0
Il-10-/-→Ldlr-/-
Clec9a-/-Il-10-/-→Ldlr-/-

Figure 3


A
CD45.1
CD45.2
Spleen
***
%
CD45.1
CD45.2


B
Dc Clec9a mRNA
***
arbitray units
Cont
Clec9a-/-
Cont
Clec9a-/-
CD8α+
CD8α-


C
CD8α+DC-Cont
CD8α+DC-Clec9a-/-

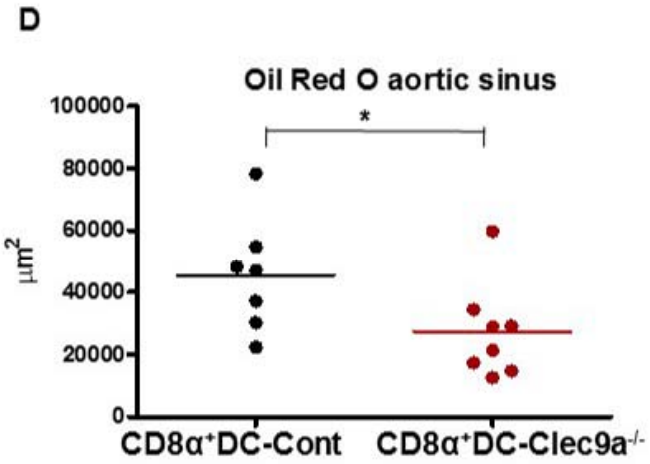

D
Oil Red O aortic sinus
*
µm2
CD8α+DC-Cont
CD8α+DC-Clec9a-/-

Figure 4

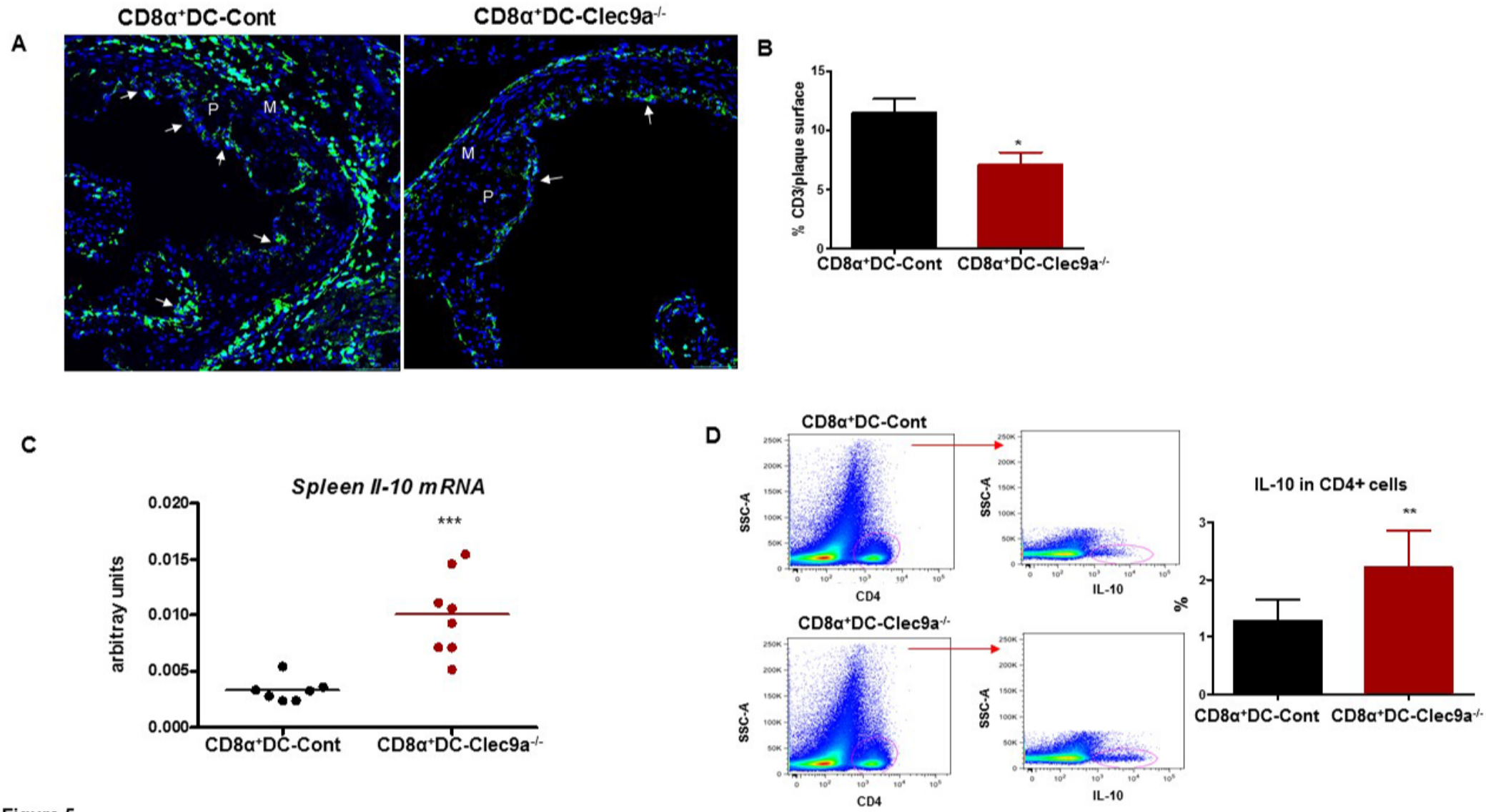

A
CD8α⁺DC-Cont
CD8α⁺DC-Clec9a⁻/⁻
P
M
M
P
B
% CD3/plaque surface
15
10
5
0
*
CD8α⁺DC-Cont
CD8α⁺DC-Clec9a⁻/⁻
C
Spleen Il-10 mRNA
arbitray units
0.020
0.015
0.010
0.005
0.000
***
CD8α⁺DC-Cont
CD8α⁺DC-Clec9a⁻/⁻
D
CD8α⁺DC-Cont
SSC-A
CD4
IL-10
CD8α⁺DC-Clec9a⁻/⁻
IL-10 in CD4+ cells
%
**
CD8α⁺DC-Cont
CD8α⁺DC-Clec9a⁻/⁻

Figure 5

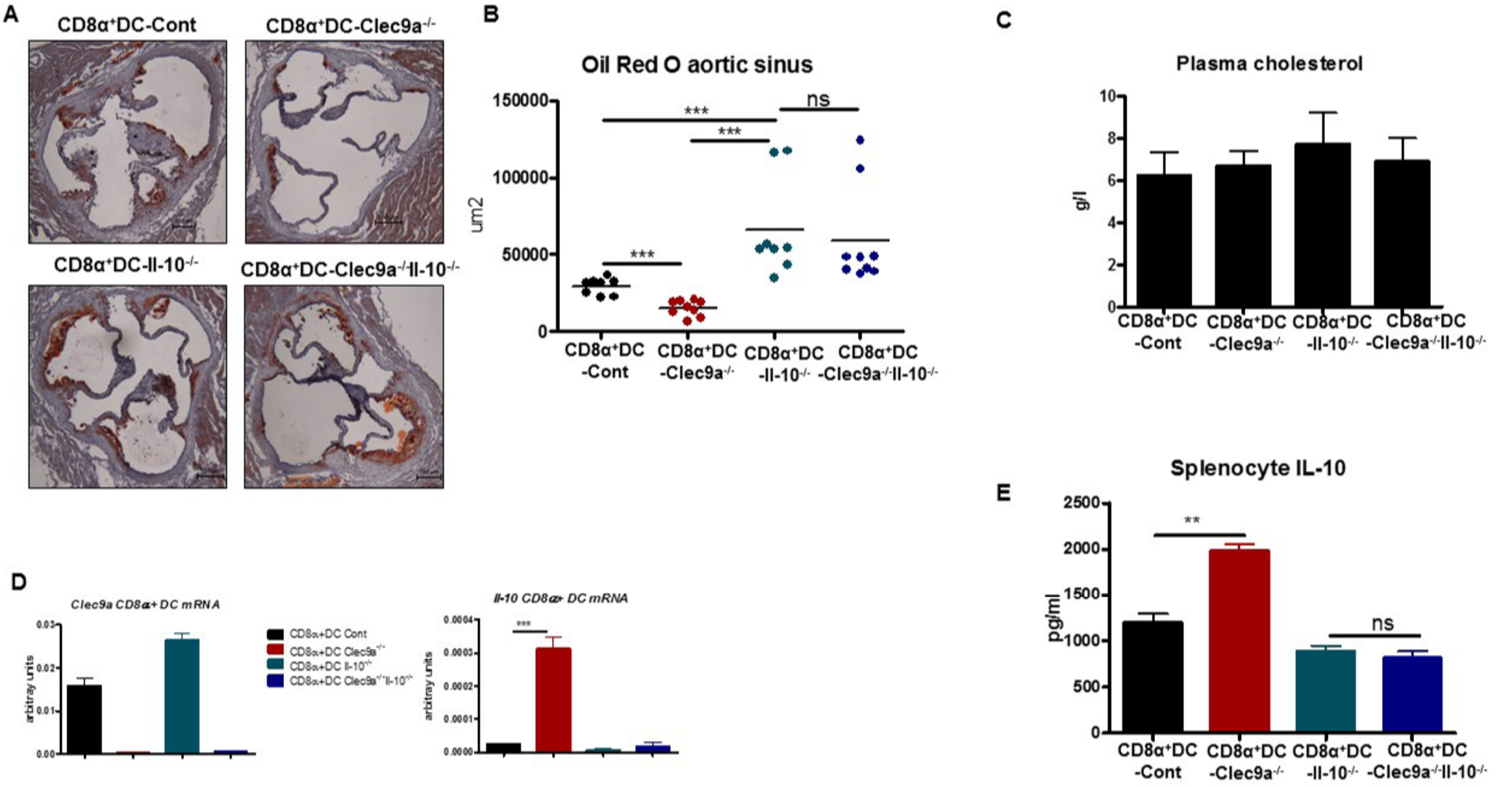

A
CD8α+DC-Cont
CD8α+DC-Clec9a-/-
CD8α+DC-Il-10-/-
CD8α+DC-Clec9a-/-Il-10-/-
B
Oil Red O aortic sinus
um2
150000
100000
50000
0
***
***
***
ns
CD8α+DC -Cont
CD8α+DC -Clec9a-/-
CD8α+DC -Il-10-/-
CD8α+DC -Clec9a-/-Il-10-/-
C
Plasma cholesterol
g/l
10
8
6
4
2
0
CD8α+DC -Cont
CD8α+DC -Clec9a-/-
CD8α+DC -Il-10-/-
CD8α+DC -Clec9a-/-Il-10-/-
D
Clec9a CD8α+ DC mRNA
arbitray units
0.03
0.02
0.01
0.00
CD8α+DC Cont
CD8α+DC Clec9a-/-
CD8α+DC Il-10-/-
CD8α+DC Clec9a-/-Il-10-/-
Il-10 CD8α+ DC mRNA
arbitray units
0.0004
0.0003
0.0002
0.0001
0.0000
***
E
Splenocyte IL-10
pg/ml
2500
2000
1500
1000
500
0
**
ns
CD8α+DC -Cont
CD8α+DC -Clec9a-/-
CD8α+DC -Il-10-/-
CD8α+DC -Clec9a-/-Il-10-/-

Figure 6